Black mulberry fruit extract alleviates streptozotocin-induced diabetic nephropathy in rats: targeting TNF-α inflammatory pathway

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Keywords
cyanidin glycosides; diabetic nephropathy; Morus nigra fruit extract; oxidative stress; phenolic compounds; proinflammatory cytokines

Abstract

Objectives This study was designed to investigate the effect of *Morus nigra* fruit extract in retarding the progression of diabetic nephropathy in streptozotocin (STZ)-induced diabetic rats.

Methods Diabetic male Wistar rats were injected with black mulberry fruit extract (BMFE) at doses of 150 and 300 mg/kg body weight. After 4 weeks, microalbuminuria was estimated in addition to serum concentrations of glucose, insulin, creatinine and albumin.

Key findings The study revealed a significant amelioration of all the measured parameters in diabetic animals. In addition, MDA, lipid peroxide levels and catalase activity were also improved. The histopathological examination of kidney tissues revealed significant improvement of the pathological changes and glomerular sclerosis in diabetic rats treated with BMFE. Treated rats showed downregulation of TNF-α, vascular cell adhesion molecule-1 (VCAM-1) and fibronectin mRNA expression.

Conclusion The ameliorative effect of BMFE on diabetic nephropathy is not only through its potent antioxidant and hypoglycaemic effects but also through its downregulation of TNF-α, VCAM-1 and fibronectin mRNA expression in renal tissues of diabetic-treated rats. Therefore, BMFE as dietary supplement could be a promising agent in improving diabetic nephropathy.

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by decreased antioxidant enzyme activity causing oxidative stress-induced damage to pancreatic beta cells. As a result, insulin secretion is decreased and the subsequent hyperglycaemia further exacerbates the oxidative stress conditions through the generation of reactive oxygen (ROS) and nitrogen species (RNS). Diabetic nephropathy is considered the most common cause of renal failure among diabetic patients. Growing evidence indicates that pathogenesis of DM is widely related to the activation of the innate immune system and the presence of a low-grade chronic inflammation. It is currently believed that macrophages infiltrate the diabetic kidney in the setting of subclinical chronic inflammation with subsequent production of proinflammatory cytokines that ultimately results in diabetic kidney injury. The most important inflammatory cytokines induced in this setting are TNF-α, in addition to increased activation of transcription factor NF-κB, which contributes also to the progression of DM. TNF-α may cause direct cytotoxicity to renal cells, inducing direct renal injury, apoptosis and necrotic cell death. It can also produce alterations of intraglomerular blood flow and reduction of glomerular filtration as consequence of the disequilibrium between factors promoting vasoconstriction.
and vasodilation.\textsuperscript{[9]} In addition, TNF-\(\alpha\) is able to directly induce ROS formation by renal cells.\textsuperscript{[10]} resulting in alterations of the glomerular capillary wall and consequently increased albumin permeability.\textsuperscript{[11]} Many studies reported that the proinflammatory cytokines such as monocyte chemoattractant proteins-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and transforming growth factors-\(\beta\)-1 (TGF-\(\beta\)-1) are elevated in diabetic kidneys leading to inflammation state, glomerulosclerosis and tubulointerstitial fibrosis.\textsuperscript{[12-14]}

Fruits, leaves and roots of \textit{Morus} species, especially black mulberry, have been used traditionally as anti-diabetic herbal medications.\textsuperscript{[15]} \textit{Morus nigra} L. fruit, also known as black mulberry fruit (BMF), is considered the healthiest fruit among mulberry species due to its high content of flavonoids, anthocyanins and other phenolics.\textsuperscript{[16]} The leaf and fruit extracts of white mulberry (\textit{M. alba}), black mulberry or their combination have been reported to have potential antiobesity and anti-diabetic effects.\textsuperscript{[17-18]} Additionally, the fruit has a free radical scavenging effect\textsuperscript{[19]} which is mostly attributed to its high content of phenolic compounds especially anthocyanins such as cyanidin-3-\(\beta\)-D-glucopyranoside which previously showed neuroprotective effects against cerebral ischaemia.\textsuperscript{[20]} Among mulberry fruits, black mulberry fruits were estimated to have higher contents of phenolics and flavonoids than other mulberry fruits.\textsuperscript{[16]} Moreover, the anthocyanin-rich fraction of white mulberry fruit (\textit{M. alba}) extract improved insulin resistance and protected the hepatocytes against oxidative stress during hyperglycaemia.\textsuperscript{[21]} Additionally, three different extracts of BMF have been reported to have a protective action against peroxidative damage of biomembranes and biomolecules.\textsuperscript{[22]} In addition to its anti-hyperglycaemic effect in diabetic rats, BMF aqueous extract caused an improvement in kidney functions which was directly linked to its ability for scavenging free radicals and inhibiting renal aldose reductase activity in diabetic rats.\textsuperscript{[23]} In a separate study, administration of BMFE at 800 mg/kg prevented kidney tissue damage in diabetic rats.\textsuperscript{[24]} The biological benefits of BMF intake on diabetic and non-diabetic subjects were reported by Abdalla (2006), who considered BMF an excellent food supplement for anaemic, diabetic and hypertensive patients.\textsuperscript{[25]}

Therefore, the current study was conducted for better understanding of the molecular mechanisms involved in the pathogenesis of diabetic nephropathy and how it may be affected by black mulberry fruits extract, BMFE. In a continuation to our interest in Egyptian folk medicine as remedies for the treatments for diabetes as specific objective\textsuperscript{[26]} and establishment of Egyptian herbal Monographs as broad objective, we investigated the effect of BMFE in a rat model for diabetic-induced kidney damage. Although previous investigations have confirmed the protective effect of BMF intake against kidney damage in diabetic rats, most studies have attributed this effect to the antioxidant properties of BMF. Herein, we aim to investigate the modulating effect of BMFE in the pathogenesis of diabetic nephropathy focusing on inflammatory cytokines, including TNF-\(\alpha\) and markers of endothelial dysfunction such as VCAM-1 and fibrosis-related proteins as fibronectin. Our findings here not only validate the protective potential of BMFE against diabetic nephropathy by outlining its underlying mechanism but also differentiate BMFE from other natural antioxidants consumed by the general public to be specifically beneficial for diabetic patients.

\section*{Methods}

\subsection*{Chemicals}

Authentic anthocyanin samples of cyanidin-3-\(\beta\)-glucoside and cyanidin-3-\(\beta\)-rutinoside for high-performance liquid chromatography (HPLC) analysis were purchased from Phytoplan company (Germany). Streptozotocin (STZ), chloroform and isopropanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Authentic samples for HPLC analysis of flavonoids rutin, quercetin, quercetrin, catechin, kaemferol, naringin and phenolic acids chlorogenic, gallic, caffeic, ellagic and cinnamic acids were purchased from Sigma-Aldrich, Germany and were kindly supplied by Food Technology Research Institute, Giza, Egypt. TRizol was obtained from Genetix Brandi Co. (Genetix Biotech Asia Pvt. Ltd., New Delhi, India). Quantifast SYBR Green PCR Master Mix unit was provided by QIAGEN (Hilden, Germany). HiSenScript\textsuperscript{\textsuperscript{(\textsuperscript{\textsuperscript{TM}}\textsuperscript{\textsuperscript{-}}\textsuperscript{\textsuperscript{-}})}} cDNA synthesis kit was purchased from iNtRON Biotechnology Company. The other reagents were of analytical, high-performance liquid chromatography (HPLC), or the best accessible pharmaceutical grades.

\subsection*{Plant material}

Ripe fruits of \textit{M. nigra} were collected in April 2014 from a private garden along Alexandria Cairo Desert Road and authenticated by Dr. Mohammed El- Gebaly, Department of Botany, National Research Center. A voucher specimen (#2013.05.15) of the plant was kept at the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

\subsection*{Preparation of the extract}

Fresh fruits of \textit{M. nigra} L. (1 kg) were homogenized at room temperature for 10 min two times each with four litres of 80% using IKA T-50 Ultra-Turrax homogenizer at 4000 rpm (IKA, Staufen, Germany). The combined extracts
were filtered and evaporated to dryness using rotary evaporator at 45°C to yield 160 g of back mulberry extract (BMFE) as dark purple residue.

**Chemical analysis of BMFE**

*Estimation of total phenolics (TP) and total flavonoid (TF) content*

Total flavonoid content was determined spectrophotometrically through complexation with AlCl₃ reagent and was estimated as mg quercetin equivalent/g (mgQE/g) based on a pre-established calibration curve of quercetin.[27] Total phenolics were determined using Folin–Ciocalteu method and expressed as mg Gallic acid equivalent per gram extract (mgGAE/g) based on a calibration curve of Gallic acid.[27]

**HPLC analysis of anthocyanins**

Samples of BMFE were dissolved in 20% methanol in water using sonication at room temperature for 5 min. The extract was filtered through a membrane filter (0.2 µm pore size); then, 20 µl was injected into an Agilent series 1100 HPLC system with UV detector set at 530 nm. Chromatographic separation was achieved on chromosphere RP-18 column (250 × 4.6 mm, 5 µm) using mobile phase of 0.5% acetic acid (solvent A) and acetonitrile containing 0.5% acetic acid (solvent B) in a gradient manner: 0 min 100% A, 15–18 min 80% A/ 20% B, 18–27 min 100% B and 27–30 min 100% A. All HPLC runs were done in triplicate. Linearity and precision were evaluated by establishing a standard calibration curve of cyanidin-3-O-glucoside (Phytoplan company, Germany) at concentration range of 10–75 µg/ml.

**HPLC analysis of flavonoids and phenolic acids**

Chromatographic separation targeting both flavonoids and phenolic acids was achieved using HPLC Agilent 1200 equipped with UV detector which was set at 280 nm for detection of phenolic acids and 330 nm for detection of flavonoids. Chromatograms used for identification of flavonoids were acquired using a mobile phase consisting of 50 µl H₂PO₄, pH 2.5 (solution A) and acetonitrile (solution B) as follows: isocratic elution 95% A/5% B, for 5 min followed by linear gradient to 50% A/50% B over 20 min and isocratic elution 50% A/50% B for the final 5 min. Rutin was quantified in the extract used a calibration curve of serial dilutions of rutin (0.6–50 mg/ml).

Chromatograms used for identification of phenolic acids were obtained using a binary gradient consisting of A (aqueous acetic acid 2.5%), B (acetonitrile) with the following gradient: at 0 min: 95% A/5% B; at 5 min: 90% A/10% B; at 10 min: 70% A/ 30% B; at 25 min, 100% B then holding isocratic until 30 min. The solvent flow rate was 1 ml/min. The injection volumes were 5 µl.

**DPPH radical scavenging assay**

The antioxidant potential of BMFE was assessed using a modified quantitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay as described previously by Ref. [28]. All experiments were carried out in triplicate. The scavenging activity of the samples was calculated as a percentage of free radical inhibition (I %) according to this formula:

\[
I\% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

**Biological study**

**Animals**

Eighty male Wistar rats, weighting 170–200 g, were purchased from a private Lab animal farm (Tanta, Egypt) and were kept in animal house of Faculty of Veterinary Medicine, Kafrelsheikh University and housed in well-ventilated plastic cages. All rats were kept on a 12 : 12-h light/dark cycle. Rats were kept for two weeks for acclimatization before treatment. All animals received a commercial standard diet (El-Nasr Co, Cairo, Egypt) and water ad libitum. The experiment was carried out according to the guideline of the institutional animal care at Kafr-Elsheikh University (13/2/2018) and following ‘Guide for the Care and Use of Laboratory Animals’ issued by United States National Research Council, 2011.

**Induction of diabetes and experimental protocol**

Diabetes was induced in overnight-fasted rats by a single intraperitoneal (IP) injection of freshly prepared STZ (50 mg/kg, in 0.09 M cold citrate buffer, pH 4.8).[26] The STZ-injected rats were supplemented with 5% glucose (10 ml/rat) for 24 h to avoid initial hypoglycaemic death. Rats of the control group were injected IP with equal volume (0.5 ml per rat) of the vehicle (0.1 M citrate buffer, pH 4.5). Rats were tested for successful induction of diabetes at 2, 5 and 7 days after STZ injection. Blood samples were withdrawn from the tails to determine blood glucose levels using a glucometer (Bionime GM300). Only rats with blood glucose levels ≥250 mg/dl were considered diabetic and were enrolled in this study. Rats were randomly assigned into four experimental groups (n = 20), and each group was subdivided into four replicates (n = 5). The treatment protocol was lasted for 4 weeks and was as follows:

Group-1: (control group) animals of this group were injected once with 0.5 ml of 0.1 M citrate buffer and 0.5 ml
saline daily; Groups 2–4: received a single IP injection of STZ (50 mg/kg body weight in citrate buffer). Group-2: (negative group) diabetic animals without any treatment and received 0.5 ml saline daily; Group-3: diabetic animals received fruit extract (BMFE, 150 mg/Kg); Group-4: diabetic animals received fruit extract (BMFE, 300 mg/Kg). Animals of groups 3 and 4 received a daily single dose of BMFE orally by stomach tube for 4 weeks. The doses used in the current study were based on a preliminary non-published data and lower than those used by the same authors\(^{26}\) on \textit{M. nigra} leaves (200 and 400 mg/kg).

**Blood samples**

After 4 weeks (end of the experiment), rats were anaesthetized with thiopentone sodium (50 mg/Kg) IP according to Ref. [29]. Two blood samples were collected from the retro-orbital venous plexus of each rat. The first sample was collected in Eppendorf tubes containing heparin then were centrifuged at 3000 g for 20 min for plasma separation and kept at \(-80^\circ\mathrm{C}\) for catalase (CAT) assessment. The second sample was allowed to coagulate and then centrifuged at 3000 g for 15 min. The clear sera were collected kept at \(-80^\circ\mathrm{C}\) for biochemical analysis of serum glucose, creatinine, albumin, malondialdehyde (MDA) and insulin.

**Urine samples**

Urine samples were gathered on day 28 of the experiment at morning by manual massage of the urinary bladder. Collected urine was centrifuged at 3000 g for 20 min for expelling any debris, then transferred into clean dry microtubes and kept at \(-80^\circ\mathrm{C}\) till being used for estimating microalbuminuria (Biosystems, Barcelona, Spain; 22324) concentration.

**Tissue samples**

Rats were sacrificed by decapitation at the end of the experiment under mild anaesthesia utilizing thiopentone sodium (50 mg/kg body weight) by IP injection. Right kidneys were excised, cleaned and washed with cold saline and rapidly snap-frozen in liquid nitrogen and preserved at \(-80^\circ\mathrm{C}\) before RNA extraction and real-time PCR, and the left kidney tissues were settled in 10% formalin for histopathological investigation.

**Biochemical assays**

Blood glucose concentration was measured spectrophotometrically utilizing commercially diagnostic available kits (Spinreact, Girona, Spain; MD41011); serum creatinine was estimated utilizing creatinine units (BioSystem, Cairo, Egypt; 11502); serum albumin was measured using commercially available albumin kits (Diagnostics ELITech Company, Cairo, Egypt; 210001). Microalbuminuria was estimated using microalbumin kits (BioSystem, Egypt, 22324). Activity of serum lipid peroxide (MD 25 29) and plasma catalase was estimated by commercial kits (Biodiagnostic, Cairo, Egypt, CA 25 17). Rat insulin was assessed by ELISA kits (Crystal Chem. CO., Elk Grove Village, IL, USA, 90010). All methodology was done following instructions provided by the manufacturer.

**Histopathology**

Tissue specimens were collected from left kidneys and rapidly fixed in 10% neutral buffered formalin solution for at least 24 h. Fixed samples were processed through the conventional paraffin embedding technique including dehydration through ascending grades of ethanol, clearing in three changes of xylene and melted paraffin, and ending with embedding in paraffin wax at 60°C. Paraffin blocks were prepared, from which 5 µm-thickness was obtained. These sections were stained with H&E and periodic acid Schiff (PAS) according to Ref. [30]. Histopathological changes were examined under the light microscope Leitz DMRB (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). Pictures were taken with an advanced camera (Leica DFC 295, Leica Microsystems Wetzlar GmbH) at the amplification of \( \times 200 \) and \( \times 400 \) for H&E and PAS staining, respectively. The pathological grade was determined according to 16-point scale involved the following items: glomerular atrophy, hypertrophy, renal tubular degeneration and inflammatory cells infiltration. The normal structure indicated by zero-point, one point represented the mild lesions, two points for the moderate lesions, 3 points indicated the severe focal lesions and 4 points indicated the severe diffuse lesions.

For quantitative estimation of glomerular sclerosis, at the very least 50 glomeruli for each rat were scored randomly by different examiners on a self-decisive scale from 0 to 4,\(^{31}\) and then, the mean of glomerular sclerosis was statistically calculated.

**RNA extraction and cDNA synthesis**

The right kidney tissues were snap-frozen in liquid nitrogen and preserved at \(-80^\circ\mathrm{C}\) before RNA extraction. Total RNA was extracted using TRIzol method following the manufacturer’s protocol (GeneZOl\textsuperscript{TM} RNA extraction reagent). RNA was broken up in RNase-free water, and its quality was measured utilizing a NanoDrop spectrophotometer (NanoDrop 2000c; Thermo Scientific, Waltham, MA, USA). Complementary DNA was blended utilizing the HiSenScrip\textsuperscript{TM}-[-] cDNA synthesis kit (iNtRON Biotechnology Company, Seongnam, Korea) as per the manufacturer’s instructions. Complementary DNA was incorporated by
mixing 10 µl of 2× RT reaction solution, 1 µl of enzyme mix solution, 1 µg of RNA and completed by RNase-free water to 20 µl total volume. The mixture was incubated at 50°C for 30 min and 85°C for 10 min.

Real-time polymerase chain reaction (PCR) gene expression analysis

The gene expression levels in kidney tissue were determined using real-time PCR using SYBR green qRT-PCR. The primers were formulated by Macrogen Co and are listed in (Table 1). PCR program was as follows: an introductory denaturation at 92°C for 10 min, followed by 40 cycles of 15 s at 92°C, 30 s at 60°C and 30 s at 72°C. The differences in gene expression between the groups were calculated using the ∆∆Ct (cycle time, Ct) method normalized to β-actin and expressed as relative mRNA levels compared with the placebos.

Statistical analysis

All results are illustrated as the means ± SE. The data analysis was performed utilizing Prism software (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) was performed followed by Bonferroni’s test was used to determine the statistically significant differences among groups. Values of \( P \leq 0.05 \) were considered statistically significant.

Results

Chemical characterization of BMFE

BMFE was chemically characterized by determination of TP, TF and DPPH assays. BMFE showed high TP (13.53 mgGAE/g) but much lower TF content (3.02 mgQE/g; Table S1). This can be readily explained by the fact that anthocyanins have been reported as the major secondary metabolites in BMF.\(^{[16]}\) Due to the reported biological activity of anthocyanins,\(^{[34,35]}\) BMFE was analysed by HPLC/UV (\( \lambda_{\text{max}} = 530 \) nm), which revealed the presence of two major peaks at Rt = 14.07 and 21.81 min indicating the presence of two major anthocyanins. Upon comparison with two external standards, the two peaks were identified as cyanidin-3-O-β-glucoside (2.8 mg/g) and cyanidin-3-O-rutinoside (2.7 mg/g), respectively (Figure 1 and Figures S1 and S2). Concentrations of both compounds were determined based on the standard calibration curve of cyanidin-3-O-glucoside, and results are recorded in Table S1.

The abundance of anthocyanins, flavonoids and phenolic acids in BMFE resulted in good antioxidant activity of the extract which was determined using DPPH scavenging assay and showed IC\(_{50}\) of 1.8 ± 0.007 mg/ml.

Biological assessment of BMFE

Body mass

Body mass of STZ-diabetic rats was significantly lower than of the non-diabetic control group (\( P < 0.05 \)). In contrast, diabetic rats in groups 3 and 4 which received BMFE (150 and 300 mg/Kg, respectively) showed a significant increase in body mass in comparison with STZ-diabetic rats (Table 2).

Blood glucose and insulin

Fasting blood glucose concentrations (FBG) were assessed every week, and diabetic animals consistently showed higher blood glucose (\( P < 0.05 \)) than control rats during all period of the experiment (Figure 2). Treatment with BMFE by two doses fundamentally ameliorated the hyperglycaemia caused by STZ (Figure 2). FBG declined in diabetic animals approximately by 50% compared with untreated diabetic animals by the end of the experimental period. Furthermore, serum insulin level significantly increased by the end of 4th week by more than 50% of its normal value in the animals receiving BMFE at both doses (Table 2).

Effect of BMFE on diabetic nephropathy biochemical parameters

The effect of BMFE on the biochemical parameters related to kidney was compiled in Table 2.

Table 1 Primers and annealing temperatures used in real-time PCRs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF alpha</td>
<td>F: 5’-GACCTCACACTTAGATCCATCTC-3’ R: 3’-CCTCCTTGGATTACCATCGGCA-5’</td>
<td>60°C</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>F: 5’-GACTGGACGTTGGCATCATGTC-3’ R: 3’-AGTCTGATGAAATCAACATCGGCA-5’</td>
<td>60°C</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>F: 5’-CAGCAGCAGCTGACCTT-3’ R: 3’-TTATGGGAGATCCATGCCA-5’</td>
<td>60°C</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: 5’-TTATGACTGCAGGTC-3’ R: 3’-TAATGACTGCCAGTTTC-5’</td>
<td>60°C</td>
</tr>
</tbody>
</table>
Concentration of serum creatinine, as a renal capacity test, was significantly ($P < 0.05$) elevated in STZ-diabetic animals compared with normal control animals, but significantly diminished ($P < 0.05$) by treatment with BMFE relative to diabetic control rats and was restored nearly to their normal values at the higher dose.

Figure 1  HPLC-UV analysis of black mulberry fruit extract. HPLC chromatograms used for chemical characterization of BMFE. Different chromatographic conditions were used to target different chemical classes of secondary metabolites as detailed in Material and Methods section. (a) Chromatogram obtained for identification of flavonoids at $\lambda_{330}$, (b) chromatogram obtained for possible identification of phenolic acids at $\lambda_{280}$, (c) chromatogram obtained for identification of anthocyanin at $\lambda_{530}$, (d) example of the use of external standard to identify eluted peaks; showing HPLC chromatogram of an external reference standard cyanidin-3-rutinoside obtained at the same condition as (c). Arabic numerical represent eluted peaks which were identified by use of external standard; 1: naringin, 2: rutin, 3: quercetin, 4: quercetin, 5: gallic acid, 6: chlorogenic acid, 7: ellagic acid, 8: cinnamic acid, 9: cyaniding-O-glucoside, 10: cyanidin-O-rutinoside. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2  Effect of Morus nigra fruit extract on biochemical parameters by the end of fourth week

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>M.N (150 mg/kg)</th>
<th>M.N (300 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>220 ± 6.47$^a$</td>
<td>143.7 ± 5.55$^b$</td>
<td>201 ± 4.80$^b$</td>
<td>206 ± 5.96$^b$</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>15.8 ± 0.75$^a$</td>
<td>1.83 ± 0.41$^c$</td>
<td>7.86 ± 0.44$^b$</td>
<td>9.22 ± 0.34$^b$</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.65 ± 0.09$^b$</td>
<td>1.57 ± 0.11$^a$</td>
<td>0.79 ± 0.04$^b$</td>
<td>0.69 ± 0.11$^b$</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>6.28 ± 0.67$^a$</td>
<td>2.37 ± 0.48$^a$</td>
<td>5.21 ± 0.63$^b$</td>
<td>4.98 ± 0.41$^b$</td>
</tr>
<tr>
<td>Micro albuminuria (mg/dl)</td>
<td>0.91 ± 0.11$^c$</td>
<td>2.83 ± 0.47$^a$</td>
<td>1.12 ± 0.12$^b$</td>
<td>1.42 ± 0.31$^b$</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 15/group). Means in the same raw with different superscript (a, b) are significantly different ($P < 0.05$). M.N, Morus nigra extract.

Concentration of serum creatinine, as a renal capacity test, was significantly ($P < 0.05$) elevated in STZ-diabetic animals compared with normal control animals, but significantly diminished ($P < 0.05$) by treatment with BMFE relative to diabetic control rats and was restored nearly to their normal values at the higher dose. Similarly,
serum albumin concentration was improved significantly ($P < 0.05$), both doses showed values comparable to the healthy control group (Table 2). Microalbumin concentration in urine of STZ-diabetic animals was significantly increased ($P < 0.05$) in comparison with normal control (Table 2), but were significantly ($P < 0.05$) reduced by both doses of BMFE.

Antioxidant effect of BMFE

The effect of BMFE on lipid peroxidation and the antioxidant status was assessed by measuring MDA level and catalase activity. The antioxidant capacities were found to have significant differences, in which catalase activity was significantly ($P < 0.05$) dropped in diabetic rats than normal rats, as shown in Figure 3. Conversely, diabetic rats had significantly ($P < 0.05$) higher MDA levels compared with the healthy control rats. On the other hand, oral supplementation of BMFE significantly ($P < 0.05$) restored the dropped CAT activity and declined the elevated MDA levels by both doses of BMFE compared with diabetic rats.

Effect of BMFE on renal histopathology

Histopathological analysis of the kidney sections of normal, diabetic and treated groups was demonstrated in Figure 4a–h. The normal rats showed normal glomerular and tubular structures (Figure 4a). The kidney of diabetic rats
Effect of Morus nigra in nephropathic rats Tarek Kamal Abouzed et al.
revealed an extensive pattern of progressive nephropathy manifested by glomerular, tubular and renal interstitial injury. There was marked glomerular atrophy associated with a marked degree of glomerulosclerosis and dilated bowman’s space (Figure 4b). In addition, some glomeruli showed glomerular hypertrophy due to the expansion of the mesangial matrix. In addition, glomerulo-interstitial nephritis was evident, which characterized by periglomerular, peritubular and interstitial infiltration of mononuclear cells especially macrophages and lymphocytes (Figure 4b). The renal tubules in the cortico-medullary junction showed marked dilatation, which referred to hydrenephrosis extending to the cortex. Congestion of the peritubular blood vessels was also noticed.

Diabetic animals treated with BMFE at a dose of 150 mg/kg showed a marked decrease in glomerular sclerosis, with renal tubules within the normal limits (Figure 4c). The treated animal with 300 mg/kg of BMFE showed marked decrease in both glomerular sclerosis and attenuation of glomerular hypertrophy (Figure 4d). PAS staining revealed a mild glycogen deposition around the glomerular tufts (Figure 4e) in the normal rat. While the STZ-diabetic rats showed marked glomerular deposition of sclerotic materials (Figure 4f), interestingly, the diabetic animals treated with BMFE (150 mg/kg) showed a decrease in the glomerular sclerosis grade (Figure 4g) and marked decrease in glomerular sclerosis to the normal pattern in the diabetic animal treated with a dose of 300 mg/kg (Figure 4h).

Quantitative scoring of pathological lesions was markedly increased in diabetic animals in comparison with the normal group (P > 0.005) and decreased in a dose-dependent manner on treatment with BMFE in comparison with the diabetic group (P < 0.05; Figure 4i). Similarly, results were observed in sclerosis grade of pathological grade in diabetic rats in comparison with the control group (P < 0.05), Figure 4k.

The histopathology study revealed the accelerated mesangial expansion characterized by an increase in PAS-positive mesangial matrix area in diabetic animal relative to the normal rats which showed normal renal glomeruli and tubules (Figure 4a and 4b). Administration of BMFE partially reversed the mesangial matrix, the glomerulus contained less PAS-positive matrix material (Figure 4c and 4d).

mRNA expression of TNF-α, VCAM-1 and Fibronec-tin

The obtained qPCR results revealed significant (P ≤ 0.05) upregulation in inflammatory marker as TNF-α, endothelial dysfunction marker asVCAM-1 and fibrosis-related proteins as fibronec tin gene expression in kidney of diabetic group in comparison with the normal group. Moreover, BMFE oral supplementation at both doses of (150 or 300 mg/kg) caused significant (P < 0.05) downregulation of TNF-α gene expression compared with STZ-diabetic rats as presented in Figure 5a. Also, BMFE treatment significantly reduced the expression of the molecule of vascular cell adhesion molecule-1 (VCAM1) compared with STZ-diabetic rats (Figure 5b). Similarly, BMFE oral supplementation downregulates the expression of fibronec tin in comparison with STZ-diabetic rats (Figure 5c).

Discussion

Diabetic nephropathy (DN) is the most severe complication of DM that can result in renal failure at the end-stage. Although there are several therapeutic agents used to control diabetes, it is rare to achieve perfect glycaemic control. Medicinal plants remain major sources of medical treatment for a significant segment of the world population especially in developing countries where medicinal plants are used for glycaemic control in diabetes mellitus. This study capitalizes on the rising demand for herbal medicine and nutraceutical by investigating the effect of BMFE in diabetic rats’ model.

Black mulberry leaves, fruits and barks are used in certain areas of the world as antidiabetic remedies, and recent investigations have proved their efficacy in lowering blood glucose levels in diabetic animals and to less extent in human subjects. Phytochemical investigations of...
BMFE identified two anthocyanins: cyanidin 3-O-β-glucoside and cyanidin 3-O-β-rutinoside as the two major constituents of BMFE and juice\textsuperscript{[33,38,39]} albeit with variable concentrations based on the geographical origin and the variety used. BMFE used in this study showed the same chemical profile of anthocyanins as outlined in Figure 1 and Table S1. Our chemical investigation of BMFE identified rutin as the major flavonoid in accordance with previously published reports.\textsuperscript{[33,38]} Other phenolic compounds were identified here including quercetin, quercetin, gallic acid and chlorogenic acid have not been previously reported in black mulberry fruits but were reported in fruits and leaves of other Morus species.\textsuperscript{[40,41]}

At the end of the current study, the STZ-diabetic rats showed decrease in body weight, blood insulin and CAT activity, while serum glucose and serum MDA levels were increased compared with control healthy rats. These results are in harmony with those obtained by Motevalian and Javadpour.\textsuperscript{[42]} Our main findings revealed that treatment with BMFE increased body weight, reduced blood glucose level, increased insulin level and declined MDA level and catalase activity relative to untreated STZ-diabetic rats, which may be related to their content of anthocyanin. In another study, treatment of the diabetic rats with M. nigra leaf extract caused significant decrease in blood glucose and increased insulin level and improved antioxidant activity of diabetic rats.\textsuperscript{[43,44]} The observed body weight increases in animals treated with BMFE may be due to an increased cell use of glucose, thus preserving adipose and muscle tissue.\textsuperscript{[45]} The presence of anthocyanins, flavonoids and phenolic acids in BMFE resulted in good radical scavenging activity, as detected by DPPH assay, which offered protection against ROS and preserved macromolecules such as lipids and DNA from damage induced by oxidative stress.

Figure 5. Effect Morus nigra fruit extract (150 and 300 mg/kg) on mRNA expression. (a) TNF-α; (b) VCAM1; (c) fibronectin; the result represents the means ± SEM. *P < 0.05.
stress. By lowering lipid peroxide activity and increasing catalase activity, BMFE prevented tissue apoptosis and necrosis in pancreatic beta cells gradually restoring insulin secretion and providing better control of hyperglycaemia. These findings are in harmony with previous studies showing that BMFE and its anthocyanin-rich fraction can cause a significant decrease in blood glucose level in diabetic rats which may be explained by the presence of strong antioxidant compounds. Additionally, mulberry anthocyanins (125–250 mg/kg) were also shown to enhance hepatic/peripheral tissue glucose uptake, thus lowering glucose blood levels of Zucker-diabetic fatty rats.

Serum albumin is a marker of visceral protein content as well as a negative marker of inflammation. Hypoalbuminemia is a common problem associated with diabetes and is usually attributed to nephropathy. Higher intensity of inflammation has been linked to lower kidney function and higher levels of albuminuria which are known risk factors for cardiovascular diseases. Creatinine level in blood is an index of renal function that is considered a more sensitive marker of glomerular filtration rate. According to the current result, significant increase in serum urea and creatinine concentrations in STZ-diabetic rats compared with control rats was observed indicating a progressive renal damage. Administration of BMFE showed significant amelioration of serum creatinine and serum albumin compared with untreated diabetic rats. These results are in agreement with a previous report by Hasanaliou et al. indicating that following four weeks of administration of leaves extract to diabetic rats’ serum levels of creatinine, urea and uric acid were significantly lower as compared with non-treated diabetic rats. On the other hands, Putta et al. and Metwally et al. have suggested that consumption of anthocyanins may lower the risk of diabetes and diabetic complications such as DN. The result of the current study is also in agreement with available literature regarding the presumptive biological activity of each of cyanidin 3-glucoside and cyanidin 3-rutinoside.

Long-term hyperglycaemia is known as the driving force for glycosen accumulations in the renal tubules of diabetic rats. According to the present histopathological examination, there is amelioration of glycogen accumulation and fatty degeneration of renal tubules in diabetic rats treated with BMFE, which comes in agreement with the results reported by Rahimi-Madiseh who documented that hydroalcoholic extract of M. nigra fruit may prevent damage to the kidney tissue but at higher dose (800 mg/Kg), far from the doses used in the current study. In a similar study using water extract of green tea as an antioxidant, the diabetic-treated rats showed amelioration of the histopathological changes like fatty degeneration of renal tubule relative to diabetic rats. The protective effect of BMFE may be due to its content of antioxidant components such as cyanidin 3-glucoside and cyanidin 3-rutinoside that can reduce oxidative stress in the cells, resulting in normal structures and functions.

Reactive oxygen species (ROS) play an important role in regulating gene expression under physiological status, inducing apoptosis, transduction of growth signals and immune responses. Many studies stated that STZ disrupts balance between plasma oxidative and antioxidant system, potentiates the progression of diabetes mellitus and its complications. Hyperglycaemia initiates an increase in the release of monocyte chemoattractant protein-1 (MCP-1) in the endothelium of diabetic patients and increased the expression of the molecule of vascular cell adhesion molecule-1 (VCAM1) that may cause atherosclerosis. The endothelial dysfunction is known to be associated with the inflammatory vascular process and may lead to vascular diabetic disorders. Intracellular glucose elevation causes overproduction of adhesion molecules, which is suggested to be one of the earliest events in the process of vascular inflammation. Due to the interaction between monocytes and endothelial cells, intracellular adhesion molecule-1 and MCP-1 are upregulated by activation of nuclear factor-kappa B (NF-κB). Many studies reported that the proinflammatory cytokines such as MCP1, ICAM1 and TGF-β1 are elevated in diabetic kidneys and can thus contribute to diabetic nephropathy. It translocates from the cytoplasm to the nucleus and induces the expression of MCP1, ICAM1 and TGF-β1, leading to inflammation, glomerulosclerosis and tubulointerstitial fibrosis. The present finding showed that treatment with BMFE caused downregulation of VCAM1, TNF-α, fibronectin mRNA expression, explaining the protective effect of BMFE against DN through decreasing ROS and contributing to an increase in antioxidant activity in addition to and downregulation of gene expressions of VCAM1, TNF-α and fibronectin that play a critical role in the glomerulosclerosis and tubulointerstitial fibrosis included in DN.

**Conclusion**

Diabetic nephropathy remains a major problem despite of the great efforts to limit its developing into end-stage liver damage. Our results showed that the Egyptian BMFE rich in anthocyanins and other antioxidant compounds displayed strong antioxidant and antihyperglycaemic effect in the STZ-diabetic rat model. Administration of BMFE also succeeded in alleviation of diabetic complication on kidney tissue as a result of diabetes by targeting TNF-α inflammatory pathway via inhibiting the expression of TNF-α, VCAM-1 and fibronectin. Therefore, BMF or its products
may have a good potential in preventing DN. Thus, future studies on the effect of mulberry fruits and its products for use as dietary supplement by diabetic patients are strongly recommended.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Acknowledgement

This study was supported by a grant from the Science and Technology fund of Kafrelsheikh University, Egypt (grant no. KFs-3-13-05).

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Author contributions

E.A.S. and S.A.W. conceived the project; T.K.A., D.H.A., K.M.S., E.W.G., W.A. and M.A.K. designed and carried out experiments of the biological parts and analysed the data; S.H. and E.A.M. carried out the preparation of the plant extracts and their chemical analysis of the extracts; E.A.S., T.K.A., D.H.A., E.A.M. and S.H. wrote the manuscript. All the authors read and approved the final manuscript.
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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Chemical analysis of M. nigra fruit extract.

Figure S1. Structure of major compounds tentatively identified in BMFE using HPLC.

Figure S2. HPLC/UV analysis of anthocyanins in BMFE.